

Identification of *Histoplasma capsulatum* from Culture Extracts by Real-Time PCR

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We designed and tested a real-time LightCycler PCR assay for *Histoplasma capsulatum* that correctly identified the 34 *H. capsulatum* isolates in a battery of 107 fungal isolates tested and also detected *H. capsulatum* in clinical specimens from three patients that were culture positive for this organism.

Histoplasma capsulatum is a slow-growing, dimorphic fungus that causes disease that ranges from focal and self-limited to disseminated and rapidly fatal (15, 19). Immunocompromised individuals, particularly those with advanced AIDS, are at risk for disseminated histoplasmosis (19, 20). A variety of tests are used in the laboratory for the diagnosis of histoplasmosis, but all have limitations. Histoplasma antigen detection in urine and/or serum has a variable range of sensitivity, depending on the clinical pattern, the chronicity of the affliction, and the underlying condition of the patient (6). The sensitivity of the *Histoplasma* urinary antigen test is as high as 97% in AIDS patients with disseminated histoplasmosis but ranges from 20 to 81% in nonimmunosuppressed patients with acute pulmonary histoplasmosis (6, 18, 22, 23). Serologic testing by immunodiffusion and complement fixation also has utility, but both false-positive and false-negative results may occur. False-positive serologic tests may be seen in patients with other disseminated mycoses, while false-negative results may occur in immunocompromised individuals who are unable to produce an antibody response (20, 21). Histopathologic analysis of tissue is also useful but is dependent upon adequate sampling, the experience of the observer, and the histochemical stains used (9, 15). Unfortunately, *H. capsulatum* may be misidentified in histologic sections because of the variety of morphologically similar small yeast forms, such as *Candida glabrata* and *Sporothrix schenckii*. Culture, which is often considered the “gold standard,” is limited by the slow growth of the organism, which may take more than 20 days to grow. In addition, confirmatory tests are needed for organisms suspected to be *H. capsulatum* because saprophytic morphological mimics of the mold phase of the organism exist (3, 7, 15). Confirmatory assays include the *H. capsulatum* AccuProbe (Gen-Probe, Inc., San Diego, Calif.), exoantigen testing, and temperature-induced mycelium-to-yeast conversion. The development of a real-time rapid PCR method that could be used as an alternate method of culture confirmation and potentially to test clinical specimens directly is warranted.

The LightCycler PCR system (Roche Molecular Biochemicals, Indianapolis, Ind.) affords nucleic acid amplification and detection in a closed system in a real-time format. We designed a real-time PCR assay for the detection of *H. capsulatum* that targeted the internal transcribed spacer region of the rRNA gene complex (GenBank accession number AB055231) and used hybridization probes and fluorescent resonance energy transfer technology. We used Hcap-F (5′-TTGTCTACCGGACCTG-3′) as the forward primer and Hcap-R (5′-TTCTTCA TCGATGTCGGAAC-3′) as the reverse primer. The first hybridization probe of the pair was Hcap HP-1, which had the 3′ end labeled with fluorescein isothiocyanate; the sequence of the probe was 5′-ACGATTGGCGTCTGAGC-3′-fluorescein isothiocyanate. The second hybridization probe of the pair was Hcap HP-2, which had the 5′ end labeled with Red 640-hydroxysuccinimide ester (640) and the 3′ end phosphorylated (P) to prevent probe extension; the sequence of the probe was 640-5′-GAGAGCGATAATAATCCAGTCAAAAC-3′-P. The LightCycler hybridization kit (Roche Molecular Biochemicals) was used with 4 μM MgCl₂, the forward and reverse primers at 0.5 μM each, 0.2 μM Hp-1, and 0.4 μM Hp-2. A standard reaction volume of 20 μl was used for each LightCycler capillary tube, which consisted of 15 μl of master mix and 5 μl of organism lysate or clinical specimen DNA extract. Capillary tubes were centrifuged for 5 s at 3,000 rpm to ensure that the reaction mixture was in the bottom of the capillary tubes. The LightCycler program consisted of four consecutive phases: (i) an enzyme activation phase (10 min at 95°C), (ii) a cycling program (45 cycles of 10 s at 95°C, 10 s at 55°C, and 20 s at 72°C), (iii) a melting phase (40 to 95°C at 0.1°C/s), and (iv) a cooling phase (3 min at 40°C). The F2/F1 mode was used for both quantification and melting curve analysis, as provided by the LightCycler software. Optimization experiments were performed that examined MgCl₂ concentrations of 2, 3, and 4 μM; forward and reverse primers concentrations of 0.25, 0.5, and 1 μM; and hybridization probe concentrations of 0.2 and 0.4 μM in all possible combinations (data not shown). The optimal concentrations were those given above.

We tested the *H. capsulatum* LightCycler PCR assay on 107 cultured fungal isolates, which included 34 isolates of *H. capsulatum* (Table 1). The other fungi tested included closely related fungi (i.e., strains of *Blastomyces dermatitidis*), fungi

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TABLE 1. Cultured fungal isolates tested for *H. capsulatum* by real-time PCR

Fungus tested for	Total no. of isolates	No. PCR positive	No. PCR negative
<i>Histoplasma capsulatum</i>	34	34	0
<i>Candida</i> species ^a	16	0	16
<i>Blastomyces dermatitidis</i>	8	0	8
<i>Aspergillus</i> species ^b	6	0	6
<i>Cryptococcus</i> species ^c	5	0	5
<i>Penicillium marneffei</i>	4	0	4
<i>Trichophyton</i> species	4	0	4
Miscellaneous			
Two of each ^d	10	0	10
One of each ^e	20	0	20
Total	107	34	73

^a Isolates of *C. albicans*, *C. rugosa*, *C. krusei*, *C. lusitanae*, *C. parapsilosis*, *C. glabrata*, *C. tropicalis*, *C. guilliermondii*, and *C. lipolytica* were tested.

^b Isolates of *A. niger*, *A. flavus*, *A. fumigatus*, and *A. terreus* were tested.

^c Isolates of *C. neoformans* and *C. albidus* were tested.

^d Isolates of *Arthrographis* species, *Mucor* species, *Microsporum* species, *Penicillium* species (non-*P. marneffei*), and *Coccidioides immitis* were tested.

^e Isolates of *Sporothrix schenckii*, *Trichosporon beigellii*, *Aureobasidium* species, *Chrysomya* species, *Pithomyces* species, *Phialomonium* species, *Hormonema* species, *Trichothecium* species, *Chrysosporium* species, *Rhizopus* species, *Scedosporium* species, *Scopulariopsis* species, *Fusarium* species, *Chladosporium* species, *Scytalidium* species, *Epicoccum* species, *Ochroconis* species, *Paecilomyces* species, *Rhodotorula* species, and *Epidermophyton* species were tested.

that are similar to the tissue phase of *H. capsulatum* (*C. glabrata* and *Penicillium marneffei*), and a variety of yeasts and molds that are commonly encountered in the clinical mycology laboratory. The majority of the molds were identified by their growth characteristics, as well as their microscopic and colonial morphology. The isolates of *H. capsulatum*, *B. dermatitidis*, and *C. immitis* were confirmed by their respective AccuProbes (GenProbe Inc., San Diego, Calif.). The isolates of *P. marneffei*

were identified by the presence of a red diffusible pigment in the agar, colonial and microscopic morphology, and temperature-induced mycelium-to-yeast conversion. The yeast isolates were identified by using a combination of germ tube and urea testing, the Vitek Yeast-ID card (bioMérieux, St. Louis, Mo.), and morphology on cornmeal agar.

A pure culture of each isolate was maintained on potato dextrose agar (Becton Dickinson Biosciences, Sparks, Md.). A 0.5-mm loopful of each fungus was tested. The loopful of yeast was recovered without complication; the loopful from the mold isolates was recovered by using two loops, one to recover the fungus and the second to remove adherent agar from the hyphal mat. The test sample of each isolate was then placed into 500 μ l of a lysis buffer that has been previously described, which contained 1% Triton X-100, 0.5% Tween 20, and 10 mM Tris-HCl (pH 8.0) (13). The suspension was vortexed vigorously for 1 min, boiled for 15 min at 100°C, vortexed again, and boiled for another 15 min. Tris-EDTA buffer was used as the negative control, and a lysate of an American Type Culture Collection (ATCC) isolate of *H. capsulatum* (ATCC 38904) was used as the positive control. Of the 107 fungal isolates tested, only the 34 isolates of *H. capsulatum* were positive by either quantitation or melting curve analysis (Fig. 1). The average hybridization melting temperature for the PCR from the isolates was 62.71°C (range, 58.45 to 64.95°C); the lower melting temperature may have been due to a mutation in the probe hybridization site, but this remains speculative. All of the other fungi were negative by both the quantitation and melting curve analyses. This assay was 100% sensitive and 100% specific for the detection and differentiation of *H. capsulatum* from other cultured fungal isolates.

In addition, we tested clinical specimens from three immunosuppressed patients with culture-proven histoplasmosis. The first specimen was a bronchoalveolar lavage (BAL) sample that

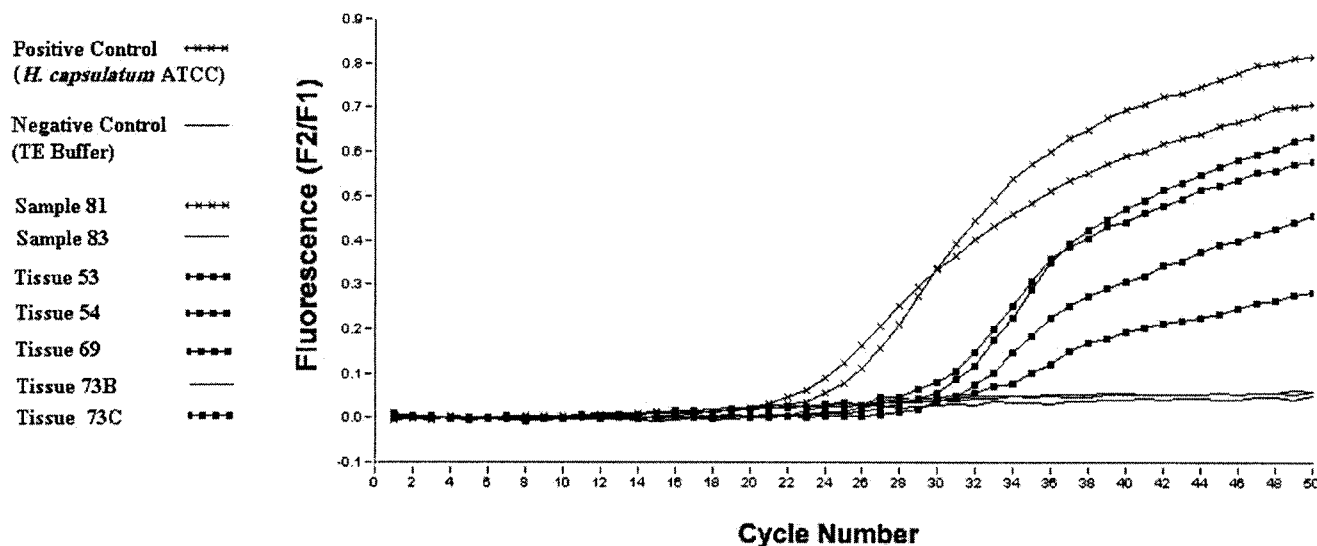


FIG. 1. PCR analysis results. The positive PCR results include (i) cultured isolates of *H. capsulatum* (x), which include the ATCC positive control strain and sample 81, and (ii) clinical specimens that were culture positive for *H. capsulatum* (black squares), which include a formalin-fixed bone marrow clot sample, a blood sample, a bronchoalveolar lavage sample, and an open-lung biopsy sample. The negative PCR results (—) depicted include (i) the negative control (TE buffer), (ii) a representative non-*H. capsulatum* culture isolate (*C. albicans*; sample 83), and (iii) the B5 fixed bone marrow biopsy sample (tissue 73B).

contained small yeast cells suspected to be *H. capsulatum*; the BAL sample was culture positive for *H. capsulatum*. The second specimen was an open-lung biopsy sample that contained small yeast cells suspected to be *H. capsulatum* in the corresponding histopathologic specimen; it was also culture positive for *H. capsulatum*. Three specimens were available from the third patient, who had disseminated histoplasmosis and a bone marrow biopsy sample positive for *H. capsulatum*. We tested a bone marrow biopsy specimen that was fixed in B5 fixative and paraffin embedded, a bone marrow clot sample that was fixed in formalin and paraffin embedded, and a peripheral blood sample that was in EDTA. All specimens were extracted with the Qiagen Tissue/Blood Extraction kit (QIAmp, Valencia, Calif.). The fixed and paraffin-embedded specimens were deparaffinized prior to nucleic acid extraction with AutoDeWax (Invitrogen Corporation, Carlsbad, Calif.). The tissue specimens were digested with the protease K included in the Qiagen kit, and nucleic acid extraction was performed in accordance with the manufacturer's guidelines. The final nucleic acid extract volume was 100 μ l. All of the clinical specimens, with the exception of the B5 fixed bone marrow biopsy sample, were positive for *H. capsulatum* by real-time PCR. The average melting temperature for the PCR from the clinical specimens was 64.68°C (range, 63.86 to 65.26°C). The failure to detect *H. capsulatum* in the specimen fixed in B5 was expected because it is well known that the B5 fixative is inhibitory to the PCR (17). Of particular interest was the positive PCR result obtained with the EDTA blood specimen from this same patient with disseminated histoplasmosis; the blood cultures that corresponded to the EDTA blood draw from which the PCR was positive were negative by lysis-centrifugation culture, but a subsequent lysis-centrifugation blood culture was positive. This patient was *Histoplasma* urinary antigen test (Specialty Laboratories, Santa Monica, Calif.) negative throughout his clinical course.

The diagnostic workup for patients suspected to have histoplasmosis includes a variety of laboratory tests, each of which has its own strengths and limitations. The culture of *H. capsulatum* from clinical specimens is usually sufficient for the diagnosis of histoplasmosis, as this organism is not a common laboratory contaminant. However, confirmatory testing of culture isolates that resemble *H. capsulatum* is necessary, since rare saprophytic molds, such as *Sepedonium* species (Linx and Greville, 1824), may produce tuberculate macroconidia (15). Isolates suspected to be *H. capsulatum* may be confirmed by temperature-induced mycelium-to-yeast conversion or exoantigen testing, but these are time consuming and technically complex, respectively. The development of the *H. capsulatum* AccuProbe (GenProbe) was a significant advance in the rapid confirmation of culture isolates suspected of being *H. capsulatum* (3, 7, 12).

PCR-based methods of detection have been described for a variety of clinically important fungi, including *Cryptococcus neoformans*, *Aspergillus* and *Candida* species, and *H. capsulatum* (1, 2, 5, 8, 11). PCR assays for *H. capsulatum* have been used to detect this fungus in experimentally infected mice and compared with standard histochemistry staining methods and in infected human tissues (1, 2). A PCR-based assay for random amplified polymorphic DNA analysis has also been described that allowed the characterization of endemic *H. cap-*

sulatum strains in Thailand (13). The genes that encode the rRNA subunits and associated genes, such as the internal transcribed spacer region, have been shown to be useful for the molecular identification of many fungi (4, 7, 8, 11, 16). A PCR assay designed to detect sequences in the 18S rRNA gene has been shown to be useful for the detection of *Aspergillus* and *Candida* species, with a sensitivity of 100% and a specificity of 98% (5). Similarly, a PCR-enzyme immunoassay method using universal fungal primers for rRNA genes was found to be highly specific for differentiating yeast-like pathogens (10).

This study demonstrates that the real-time PCR assay described is another method, available to laboratories that utilize this technology, that may be used for the confirmation of culture isolates suspected to be *H. capsulatum*. This assay was 100% sensitive and 100% specific for the differentiation of *H. capsulatum* from other cultured fungi that may be encountered in the clinical mycology laboratory. There was no cross-reactivity of this assay with genetically related fungi, such as *B. dermatitidis*, or with fungi that have forms that may be morphologically similar to the tissue form of *H. capsulatum*, such as *C. glabrata*, *S. schenckii*, or *P. marneffei*.

Detection of *H. capsulatum* in the three clinical specimens from patients with culture-proven histoplasmosis in no way suggests this test has been validated as a method for the direct assessment of clinical specimens. It does, however, suggest that more studies are needed that compare this method with other established assays used for the diagnosis of histoplasmosis. Similarly, more studies are needed to assess the usefulness of this technology for the detection and differentiation of *H. capsulatum* from morphologically similar yeast in formalin-fixed, paraffin-embedded tissues.

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